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(54) Title: PURIFICATION OF KRINGLE CONTAINING PROTEINS, AND ESPECIALLY t-PA

(57) Abstract

Kringle containing proteins capable of binding to ω-amino acids can selectively be eluted from an ion exchange column by means of ω-amino acids.

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# PURIFICATION OF KRINGLE CONTAINING PROTEINS, AND ESPECIALLY t-PA

## Field of the Invention

The present invention is related to a method for purification of kringle containing proteins from a protein solution containing such kringle containing proteins.

## Background of the Invention

Purification of proteins, especially proteins intended for medical treatment is a delicate process. The starting material for purification will typically be biological fluids, such as plasma or serum, or cell lysates or culture media if the protein is produced by culturing a cell line capable of producing the protein. Irrespective of the source of the starting material, it will normally contain a number of unwanted proteins and other materials from which the protein of interest has to be separated. The traditional purification steps include separation of cell debris or the like by filtration or centrifugation, precipitation of proteinaceous material and resolution of the proteinaceous material whereby a protein solution is obtained containing the desired protein, however still contaminated with other proteins. The protein solution will then typically be applied to suitable purification columns such as affinity chromatography or ion exchange chromatography or the like.

Affinity chromatography is well suited for small scale purification, for example for research purposes. It is, however, less attractive for commercial scale production primarily due to the high price of the column material, but also due to a non-satisfactory chemical stability of said material. The column material will be contaminated after a certain period of use and because it can not be regenerated, it will have to be replaced frequently by new material which will increase the production costs.

Affinity chromatography columns are therefore not especially well suited for initial purification steps in large scale where a lot of impurities are present.

When purifying proteins in large scale, it is advantageous in the initial steps to use a column material with low price and good chemical and physical stability. The material should also be able to withstand a high flow rate, for example 10 to 20 column volumes per hour. Such criteria will typically be fulfilled by an ion exchange column.

The traditional way to elute proteins from an ion exchange column is to change the pH or the ion strength or both. The protein of interest and the impurities will then appear in different eluates depending i.o. on their isoeletric point (pI). If the protein of interest and the impurities from which it has to be separated have an pI in the same range such elution might, however, be too unspecific because proteins with similar pI-values will to some degree be eluted together from the column. Additional time consuming and cost increasing purification steps might therefore be necessary with the risk of increased degradation and loss of the desired protein.

In EP 0256836B a method for purification of t-PA is described by which a mixture of t-PA species having different molecular weights are separated by contacting the mixture with hydroxyapatite and eluting the various fractions successively at different pH, salt concentrations or both. This method will thus suffer from the disadvantages mentioned above. The same applies to the method described in EP 0261941A wherein t-PA is purified by means of an cation exchange column and eluted by means of the salt gradient elution method. Japanese patent application No. 63-091080 describes a method for purification of t-PA by adsorbing t-PA on an adsorption carrier such as controlled pore glass (C PG), silica gel or diatomaceous earth, and elution with a solution containing an ω-amino acid. Adsorption matrixes differ substantial from ion exchange material. In adsorption chromatography the retarding forces on the matrixes are predominantly surface energy and Van der Waals forces and the separation dependes on polar and steric factors. In ion

exchange chromatography th retarding forces are predominantly electrostatic and the separation dependes upon the ionic nature of the components.

The problem encountered with the known methods might be solved if the protein of interest could be eluted from the ion exchange column by means of an eluting agent which is capable of selectively eluting the desired protein from the ion exchange column without changing the pH and ion strength in any substantial degree during the elution step. If this were possible, only the protein of interest will be eluted whereas impurities will remain on the column.

The present invention is based on the fact that it has surprisingly been shown that a certain type of amino acids, the so-called $\omega$-amino acids, have this desirable property. It has more particularly been shown that lys-plasminogen, a derivative of plasminogen, can be selectively eluted from an S-Sepharose column by means of $\epsilon$-amino caproic acid (6-AHA) in high yields.

Summary of the invention

In its broadest aspect the present invention relates to a method of purifying kringle containing proteins being capable of binding to $\omega$-amino acids from a protein solution containing a kringle containing protein, said method comprising the following steps:

a) applying the protein solution to an ion exchange column under such conditions that the kringle containing protein is bound to the column material;

b) selectively eluting the kringle containing protein by means of an $\omega$-amino acid without changing the pH and ion strength in any substantial degree; and

c) isolating the kringle containing protein from the eluate from step b) by means of further suitable purification steps.

In a more narrow aspect the present invention is related to a method of purifying plasminogen or a plasminogen derivative such as lys-plasminogen.

## Brief Description of the Drawings

The invention is further described with reference to the drawings in which

Figure 1 shows the elution profile of lys-plasminogen from S-Sepharose with 0.5 M NaCl;

Figure 2 shows the elution profile of lys-plasminogen from S-Sepharose with 3 mM 6-AHA;

Figure 3 shows a non-reduced SDS-PAGE of elution fractions from elution of lys-plasminogen with 0.5 M NaCl and 3 mM 6-AHA;

Figure 4 shows the elution profile from an affinity column (lysine-Sepharose) of the dialysed eluate from elution of lys-plasminogen with 0.5 M NaCl;

Figure 5 shows the elution profile from an affinity column (lysine-Sepharose) of the dialyzed eluate from elution of lys-plasminogen with 3 mM 6-AHA;

Figure 6 shows a non-reduced SDS-PAGE of various elution fractions from the lysine-Sepharose column elution profiles shown in Figure 4 and Figure 5, respectively; and

Figure 7 shows the result from a plasmin analysis of lys-plasminogen further purified by means of the lysine-Sepharose column.

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

Plasminogen: By plasminogen is generally meant human glu-plasminogen.

Plasminogen derivatives: This term will cover various degraded or mutated forms of plasminogen including lys-plasminogen which have retained at least one of the five kringle domains, preferably more than three and most preferred all five kringle domains. Mutation will include replacement of one or more of the amino acid residues in the native molecule or deletion or addition of amino acid residue with the purpose of modifying the properties of the molecule in a desired way.

ω-amino acids: This term means amino acids with a free amino group linked to the distal carbon atom with respect to the carboxylic acid group.

Kringle containing proteins: With kringle containing proteins is meant proteins containing one or more so-called kringle structures known from for example t-PA and plasminogen.

Selective elution: By the expression "selective elution" is meant elution of the desired protein with non detectable amounts of the non desired proteins.

Protein solution: This term includes a biological solution or an aqueous solution containing a number of proteins including the desired protein.

Biological solutions: This means plasma, serum or fractions thereof, or solutions obtained by extraction of organs of human or animal origin.

A substantial constant pH will mean a variation of ± 1 pH unit, preferably ± 0.5 pH unit compared to the pH in the buffer before addition of the ω-amino acid.

A substantial constant ion strength will mean that the ion strength may vary within a range of ± 20%, preferably ± 10% of the ion strength in the buffer before addition of the ω-amino acid.

## Detailed description

Although the present purification method may be used for purification of kringle containing proteins from biological solutions such as plasma and serum it may preferably be used for purification of such proteins from culture media or a solution obtained by disruption of cells which produce the protein intracellularly. The cells producing the kringle containing protein might be natural cells or transformed or transfected cell lines capable of expressing an inserted DNA sequence encoding the kringle containing protein.

Ion exchange column are well known within the art. Suitable ion exchange columns are S-Sepharose® and Fractogel $SO_3$.

The ω-amino acids are preferably such containing at least 4 carbon atoms in the carbon chain between the carboxylic acid and the ω-amino group. The carbon chain may be linear or cyclic. Examples of suitable linear ω-amino acids are 4-aminobutyric acid, 5-aminopentoic acid, 6-aminohexanoic acid (ε-amino caproic acid (6-AHA)), 7-aminoheptanoic acid, 8-aminooctanoic acid, lysine and arginine. Examples of cyclic ω-amino acids are trans-4-aminomethyl cyclohexane carboxylic acid (tranexamic acid) and para-aminomethyl benzoic acid.

The method of the present invention may in principle be used for purification of any kringle containing protein which binds to ω-amino acids and is capable of being

eluted from an ion exchange column without any substantial change in pH and/or ion strength during the elution. The great benefice of this is that impurities in form of other proteins bound to the column together with the protein of interest will remain on the column because the pH or ion strength is not changed in any substantial degree. The selective elution effect of the ω-amino acids might reside in the fact that their binding to the kringle containing protein is so strong that it may change the pI of the protein or that it may cause some conformation changes of the molecule to cause liberation from the column material.

An important group of proteins which can be purified by means of the present method are the so-called kringle containing proteins (see for example L. Patthy, Blood Coagulation and Fibrinolysis 1 (1990), 153-166). These proteins contain one or more kringle structures of which many are known to bind with lysine or lysine analogues, such as trans-4-aminomethyl cyclohexan carboxylic acid and ε-amino caproic acid. Examples of such kringle containing proteins are t-PA and t-PA analogues with various modification of the finger growth factor and kringle domains and round the activation site as described in published European patent application Nos. 191,843; 201,153; 207,589; 241,209; 233,013; 240,334; 293,936; 293,934 and 292,009. Other examples are plasminogen, lys-plasminogen, apo-lipoprotein a, hepatocyte growth factor, FXII, prothrombin, uPA, and a newly discovered protein with putative tumor suppressor properties (Degen S.J.F. (1991). Abstract 20th Lindenström Lang Conference, Vingsted, Denmark, 164-166).

Although the present method primarily is intended to be used in the initial purification steps where the number of impurities is large, it may also be used in a later or final purification step where some or most of the impurities have been removed by initial ion exchange or affinity chromatography column purification or other suitable means.

The eluate from the ion-exchange column is preferably further purified by additional suitable purification steps such as dialyzation, gel filtration and affinity chromatography.

The invention will be explained in the following into further detail with reference to the purification of plasminogen.

Human plasminogen is a single-chain glycoprotein with a molecular weight of about 90,000. It circulates in plasma in a concentration of about 2 $\mu$M and can be purified from human plasma by affinity chromatography on lysine coupled to Sepharose®. The native form of plasminogen has $NH_2$-terminal glutamic acid and is termed "glu-plasminogen". The plasminogen molecule consists of 791 amino acids, with 24 disulfied bridges, 5 triple-loop "kringle" structures and a serine protease domain.

Several lower molecular weight forms of human plasminogen have been obtained as a consequence of proteolytic digestion of the native molecule. The nomenclature employed for these molecules is based upon the sequence position of the amino-terminal amino acid. Exposure of glu-plasminogen to plasmin for very short times results in cleavage of the amino terminal 77 residues from glu-plasminogen, yielding a molecule of plasminogen, lys-plasminogen with a molecular weight of approximately 83,000, which is of great importance to several properties of the fibrinolytic system.

The conversion of plasminogen into the active protease, plasmin, is catalyzed by the so-called plasminogen activators (PAs). Plasmin is considered the primary fibrinolytic enzyme in the circulation of higher vertebrates but may also play an important role in other extra-vascular proteolytic events.

Plasminogen and lys-plasminogen may be used as a thrombolytic agent (see K. Anderle et al., Haemostatis 18 (1988), 165-175).

Human plasminogen may be purified from plasma by several methods. Recent methods on the purification from human plasma are all based on affinity chromatography as described by D.G. Deutsch and E.T. Mertz, Fed.Proc. 29, 647 (1979) and Science, 170, 1095-1096 (1970). An affinity matrix (lysine Sepharose®)

is prepared by covalent coupling of the α-amino group of L-lysine to Sepharose. Plasma diluted with water is passed through a lysine-Sepharose column equilibrated with phosphate buffer (pH 7.4) at room temperature, whereupon the column is washed with phosphate buffer. Plasminogen is then eluted with 0.2 M ε-amino caproic acid (pH 7.4). The ε-amino caproic acid is removed from the plasminogen, in the cold, by gel filtration on Sephadex equilibrated with phosphate buffer.

Application of human proteins purified from plasma always involves a certain risk of virus infection. In particular the risk of HIV and hepatitis infections have become a major concern in dealing with plasma-derived human proteins.

To avoid this risk it would be advantageous to produce plasminogen by means of recombinant DNA-technology. Attempts to produce plasminogen in transfected cells have, however, revealed that intracellular plasminogen activation and subsequent degradation provide an obstacle to the production of intact recombinant plasminogen in reasonable yields. This problem has been solved by coexpression of plasminogen together with a protease inhibitor capable of inhibiting plasminogen activation, see European published patent application No. 319,944.

Lys-plasminogen or glu-plasminogen may thus be produced by coexpression of Lys- or glu-plasminogen and α-2-plasmin inhibitor, $\alpha_2$-PI, in BHK cells. Glu-plasminogen or Lys-plasminogen may also be coexpressed with α-1-antitrypsin (AAT) in transfected BHK cells or a derivation thereof. Coexpression with AAT ensures that urokinase produced by the BHK cells is inhibited by AAT. Activation of plasminogen by urokinase and plasmin-mediated proteolytic cleavage is thus diminished and high levels of intact plasminogen are obtained. Plasminogen is purified from culture medium by a two step procedure comprising an ion-exchange chromatography step followed by an affinity chromatography step on lysine-Sepharose. More specifically, the ion-exchange column (S-Sepharose) is washed and then eluted with 0.5 M NaCl. The eluate is passed through an affinity chromatography column (Lysine-Sepharose) and plasminogen is isolated by elution with 6-AHA.

As mentioned above, the transfected BHK cells will express urokinase in addition to the plasminogen. The pI of plasminogen and urokinase is 6.7 - 8.1 and 8.5, respectively. Consequently plasminogen and urokinase are both bound to the ion-exchange column, whereas coexpressed plasminogen activator inhibitor will appear in the void volume.

Although high yields of plasminogen are obtained by this method it has been found that activation of plasminogen may occur to some exent during the affinity chromatography step. This is presumably caused by the fact that the plasminogen activator inhibitor is removed in the preceding ion-exchange column purification step. When plasminogen and plasminogen activator are eluted together from the ion-exchange column (S-Sepharose), even small amounts of plasminogen activator (urokinase) in the eluate may cause activation of plasminogen into plasmin and result in a plasminogen product which contains a small amount of plasmin.

Use of 6-AHA in the first ion-exchange purification step has surprisingly turned out to ensure a selective elution of lys-plasminogen without simultanous elution of urokinase. Unintentional activation of plasminogen into plasmin in the eluate by urokinase is thus avoided and a purer product is obtained compared to elution with the salt gradient method.

## Experimental part

### Example 1

#### Purification of lys-plasminogen

Lys-plasminogen was produced by a process analogous to that described in European patent application No. 319,944.

Urokinase (uk) s creted by the BHK-cells is inhibited by α-1-antitrypsin which is coexpressed together with plasminogen. The presence of the activator could be demonstrated by an exponential product development in a chromogenic assay for plasmin. The presence of uk in the culture medium has been shown by "fibrin overlay technique" (Granelli-Piperno and Reich: J.Exp.Med. 148, p. 233-34, 1978).

A sample of culture medium is split into two equal fractions.

A comparison is made between elution from an ion exchange column, S-Sepharose, in the first step either with an 6-AHA-buffer or with 0.5 M NaCl. Each eluate is further dialysed and purificated on the lysine-Sepharose.

Experiment 1

S-Sepharose, elution vith NaCl (assay No. 910723)

Equilibration buffer: 0.02 M $NaH_2PO_4$, 0.04 M NaCl, 0.02 % Tween 80. 3 mg/l Aprotinin; pH 5.0.

Washing buffer 1 = Equilibration buffer.

Washing buffer 2 = Equilibration buffer with 0.15 M NaCl; pH = 5.5.

Elution buffer 3 = Equilibration buffer with 0.50 M NaCl and without aprotinin; pH = 5.5.

Aprotinin was added to each fraction after a sample was drawn for plasmin determination.

The column was 5 ml, 750 ml culture medium was applied and the flow was 125 ml/h.

The elution profile is shown on Figure 1.

The elution profile is rather steep. Lys-plasminogen is found in the second peak.

In Figure 1, the numbers beneath the bottom line (1, 2, and 3) indicate when the various buffers are added (1. Equilibration buffer, 2. Washing buffer (0.15 M NaCl,

pH 5.5) and 3. Elution buffer (0.5 M NaCl, pH 5.5)). The numbers above in the bottom line (22...30) refer to the fraction numbers used in the SDS-PAGE (Figure 3).

Experiment 2

S-Sepharose eluted with 6-AHA (assay No. 910724

Same experiment as above except that the column is eluted in 2 steps: The first elution is performed with a elution buffer containing 0.02 M $NaH_2PO_4$, 0.18 M NaCl, 0.02 % Tween 80 and 3 mM 6-AHA, pH 5.5.
The second elution is performed as in experiment 1.

The elution profile is shown in figure 2. Lys-plasminogen is found in the second peak. The third peak represents various impurities.

In Figure 2, the numbers beneath the bottom line (1, 2, 3 and 4) indicate when the various buffers are added (1. Equilibration buffer, 2. Washing buffer (0.5 M NaCl, pH 5.5), 3. Elution buffer I (0.18 M NaCl, 3 mM 6-AHA, pH 5.5) and 4. Elution buffer II (0.5 M NaCl, pH 5.5)). The number above the bottom line (16-42) refer to the fraction numbers used in the SDS-PAGE (Figure 3).

Figure 3 shows the nonreduced SDS-PAGE of fractions from the elution from both experiments. Very many high molecular complexes are eluted together with some aprotinin.

The lane numbers represent the following:

1. Molecular weight standard.
2. Plasma lys-Plasminogen (control)
3. Culture medium used in assay 910723
4. Fraction Fig. 1 No. 23
5. Fraction Fig. 1 No. 24
6. Fraction Fig. 1 No. 25
7. Fraction Fig. 1 No. 26

(Experiment 1)

8. Culture medium used in assay 910724
9. Fraction Fig. 2 No. 16
10. Fraction Fig. 2 No. 22
11. Fraction Fig. 2 No. 23
12. Fraction Fig. 2 No. 26
13. Fraction Fig. 2 No. 30
14. Fraction Fig. 2 No. 42
15. Molecular weight standard

(Experiment 2)

It is seen that elution with 3 mM 6-AHA (lane 10-13) results in a pure product (lys-plasminogen) free of high molecular weight complexes whereas elution with NaCl (lane 4-7) results in product contaminated with some high molecular weight impurities.

To investigate whether the isolated lys-plasminogen contains minor amounts of plasmin (due to activation by urokinase in the eluate from the ion-exchange column) a further purification was conducted by means of lysine-Sepharose. This purification step will a.o. remove aprotinin.

Both eluates were thus dialysed against equilibration buffer given below and purified by affinity chromatography on lysine-Sepharose:

Column size: 5 ml, flow: 50 ml/h.
Equilibration buffer: 0.1 M $Na_2HPO_4$, 0.02% Tween 80, 3 mg/l Apr. pH 7.4
Washing buffer 1 = Equilibration buffer.
Washing buffer 2 = Equilibration buffer with 0.15 M NaCl but without aprotinin.
The elution was performed with a gradient of 0.1 M $Na_2HPO_4$ containing 0.02% Tween 80 and from 0 to 5 mM 6-AHA, 50 ml of each.

Figure 4 shows the elution profile of the dialysed eluate from experiment 1 (NaCl).

The number beneath the bottom line (1, 2, 3 and 4) refer to the following steps:
1. Application of sample,
2. Washing buffer 1,
3. Washing buffer 2 and
4 Start of eluation gradient

The numbers above the bottom line (8, 10, 12) refer to the fraction numbers used in the SDS-PAGE (Figure 6).

Figure 5 shows the elution profile of the dialysed eluate from experiment 2 (6-AHA).

The numbers beneath the bottom line (1, 2, 3 and 4) refer to the following steps:
1. Application of sample,
2. Washing buffer 1,
3. Washing buffer 2 and
4. Start of eluation gradient.

In some cases a shoulder is seen as in Figure 4. From the literature it is known that the two glycosylation forms of plasminogen have different affinity to lysine-Sepharose.

Figure 6 which is a SDS-PAGE of elution fractions from the lysine-Sepharose column shows that the lys-plasminogen is a rather pure product considering that it has only been through 2 purification steps.

The lane numbers represent the following:

1. Molecular weight standard
2. Plasma lys-Plasminogen
3. Sample applied on Fig. 4 (from assay no. 910723 without 6-AHA, Fig. 1)
4. = 3
5. Fraction Fig. 4 No. 7
6. Fraction Fig. 4 No. 9
7. Fraction Fig. 4 No. 11
8. Sample applied on Fig. 5 (from assay no. 910724 with 6-AHA, Fig. 2)
9. = 8
10. Fraction Fig. 5 No. 7
11. Fraction Fig. 5 No. 8
12. Fraction Fig. 5 No. 9
13. Fraction Fig. 5 No. 10
14. Molecular weight standard

Figure 7 shows the results from plasmin analysis of purified lys-plasminogen which has been purified according to the invention (A), i.e. elution with 6-AHA in the S-Sepharose purification step, and when elution was made with NaCl (B). The analysis was made by means of a chromogenic substrate S2251. The assay in a standard procedure where p-NA is set free from S 2251® (H-D-Val-Leu-Lys-pNA) by the enzymatic action of plasmin, thereby developing a yellow colour measured at 405 nM. All samples were diluted to the same protein concentration before the assay. It is clearly seen that plasmin is not detectable in the product which was eluted from the S-Sepharose with 6-AHA (no colour development). When the elution was performed with NaCl the product contains plasmin showing that some activation of lys-plasminogen has occured in the eluate.

CLAIMS

1. A method for purification of a kringle containing protein capable of binding to $\omega$-amino acids from a protein solution containing such kringle containing protein, said method comprising the following steps:

a) applying the kringle containing protein solution to an ion exchange column under such conditions that the kringle containing protein is to the column material;

b) eluting the kringle containing protein by means of an $\omega$-amino acid without changing the pH and ion strength in any substantial degree; and

c) isolating the kringle containing protein from the eluate from step b) by means of further suitable purification steps.

2. A method according to claim 1, wherein the $\omega$-amino acid is chosen from the group consisting of 4-aminobutyric acid, 5-aminopentanoic acid, 6-aminohexanoic acid, 7-aminoheptanoic acid, 8-aminooctanoic acid, lysine, arginine and t-aminomethylcyclohexan-1-carboxylic acid.

3. A method according to claim 1 or 2, wherein the kringle containing protein is t-PA or a t-PA analogue.

4. A method according to claim 1 or 2, wherein the protein is plasminogen or a plasminogen derivative.

5. A method according to claim 4, wherein the plasminogen derivative is lys-plasminogen.

6. A method according to any of the previous claims wherein the $\omega$-amino acid is 6-aminohexanoic acid (6-AHA).

7. A method according to any of the previous claims wherein the eluate from step b) is subjected to an affinity chromatography.

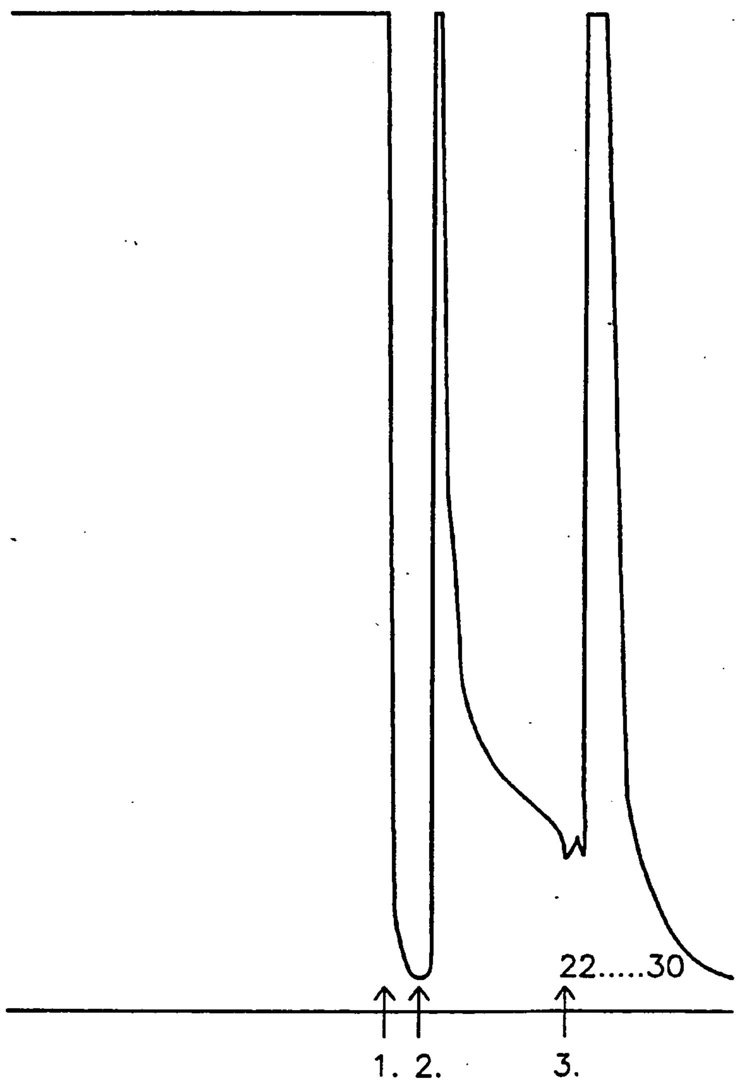



Fig. 1

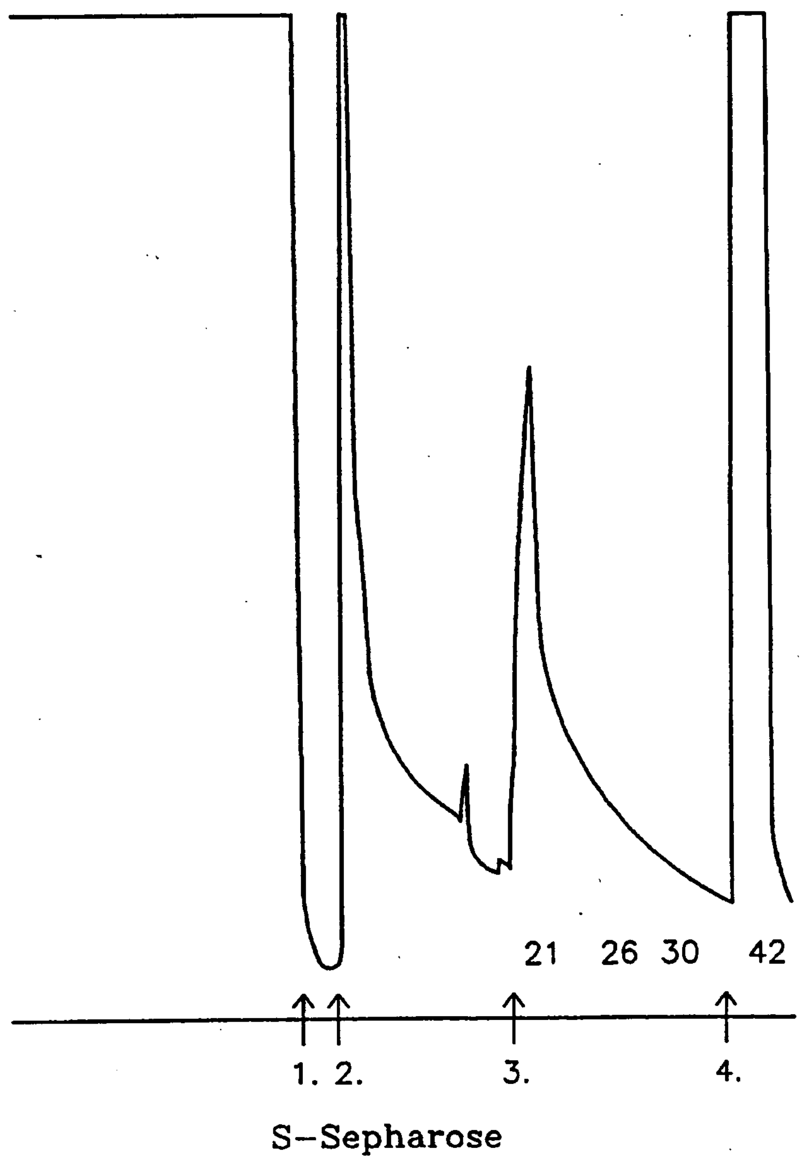
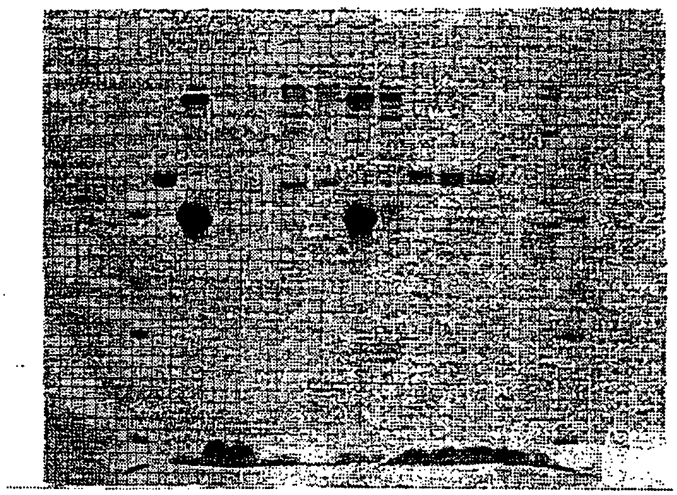



Fig. 2


1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15

SDS-PAGE, 10% gel
Non-reduced samples

Fig. 3

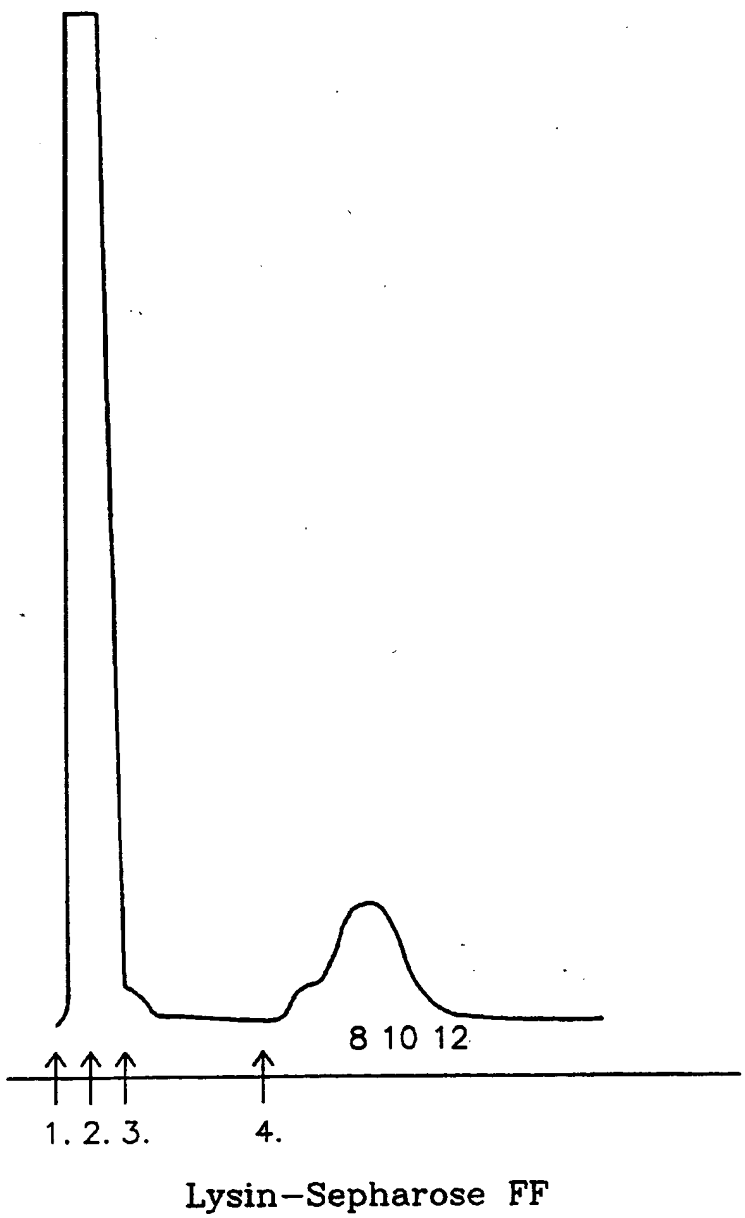
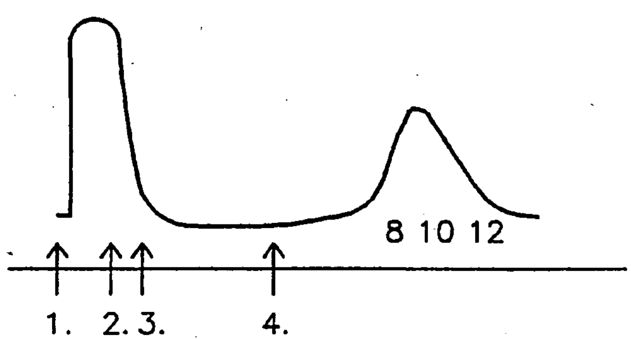
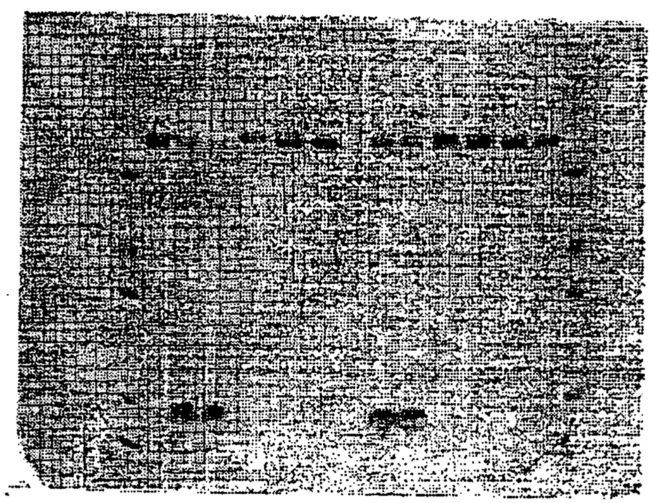



Fig. 4




Lysin−Sepharose FF

Fig. 5




SDS−PAGE analysis
10% gel,
Non−reduced samples

Fig. 6

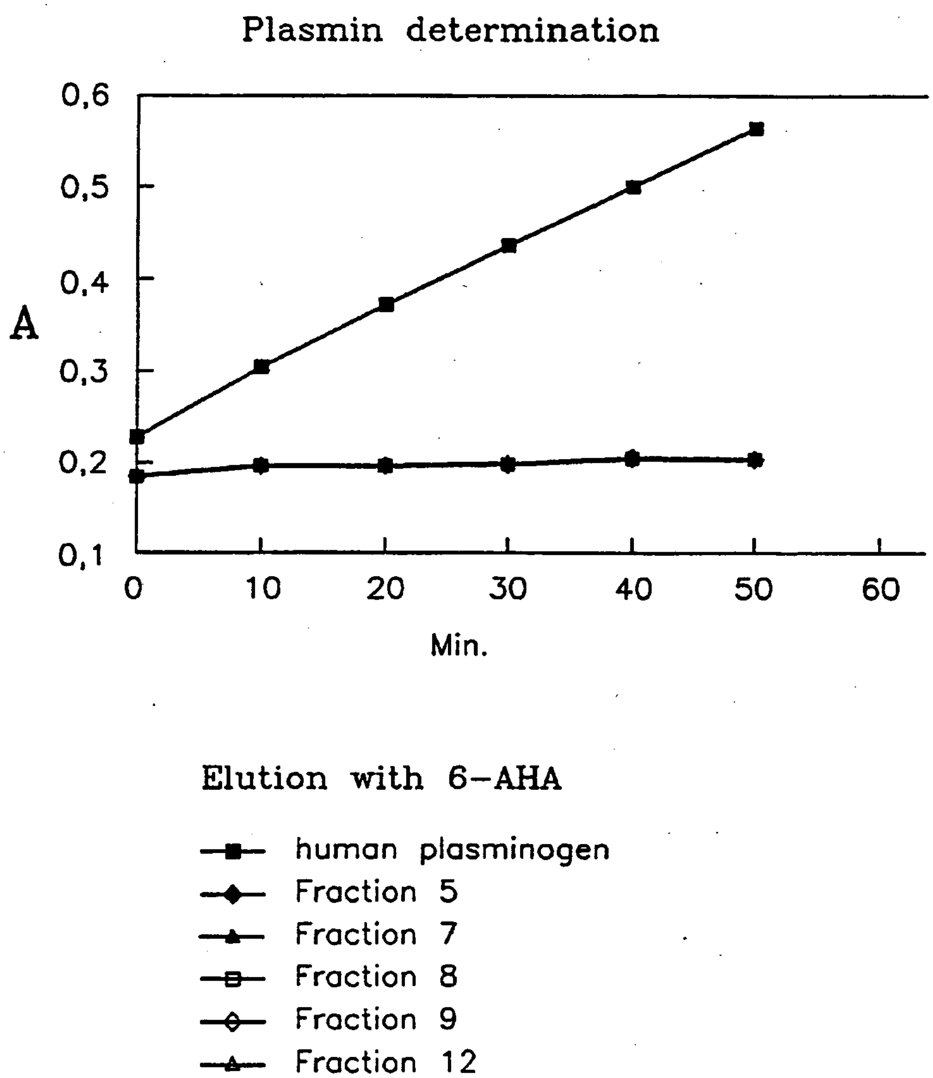
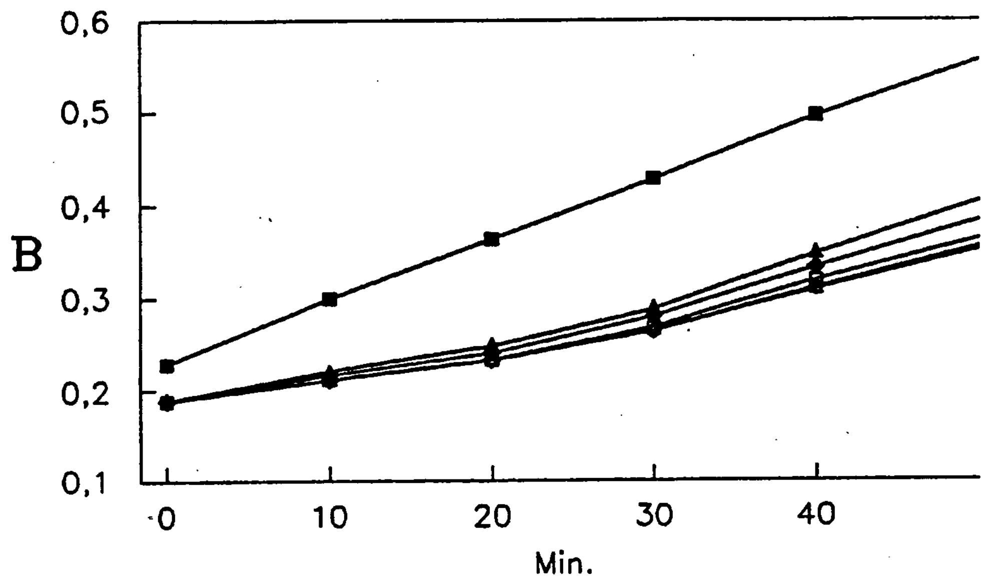

Plasmin determination
0,6
0,5
0,4
0,3
0,2
0,1
A
0
10
20
30
40
50
60
Min.
Elution with 6-AHA
human plasminogen
Fraction 5
Fraction 7
Fraction 8
Fraction 9
Fraction 12

Fig. 7

Plasmin determination




Elution with NaCl

- human plasminogen
- Fraction 5
- Fraction 7
- Fraction 9
- Fraction 11
- Fraction 13

Fig. 7 CONTINUED

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00206

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC5: C07K 3/22, A61K 37/547, C12N 9/64
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C07K, A61K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | Patent Abstracts of Japan, Vol 12,No 326, C-525, abstract of JP, A, 63-91080 (KANEGAFUCHI CHEM IND CO LTD ET AL), 21 April 1988 (21.04.88)<br>-- | 1-7 |
| A | EP, A2, 0261941 (MITSUI TOATSU CHEMICALS, INCORPORATED), 30 March 1988 (30.03.88), abstract<br>-- | 1-7 |

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
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"O" document referring to an oral disclosure, use, exhibition or other means
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"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 21 Sept 1993 | 24 -09- 1993 |
| Name and mailing address of the ISA/<br>Swedish Pat nt Office<br>Box 5055, S-102 42 STOCKHOLM<br>Facsimile No. +46 8 666 02 86 | Authorized officer<br><br>Mikael G:son Bergstrand<br>Telephone No. +46 8 782 25 00 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00206

| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|---|---|---|
| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| A | Dialog Information Services, file 154, accession no. 07610279, Medline accession no. 91129279, Menhart N. et al: "Construction, expression, and purification of recombinant kringle 1 of human plasminogen and analysis of its interaction with omega-amino acids", & Biochemistry Feb 1991, 30 (7) p 1948-57<br>--<br>-------- | 1-7 |

# INTERNATIONAL SEARCH REPORT
Information on patent family members

26/08/93

International application No.
PCT/DK 93/00206

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| EP-A2- 0261941 | 30/03/88 | SE-T3- 0261941 | |
| | | AU-B- 586355 | 06/07/89 |
| | | DE-A- 3783693 | 04/03/93 |
| | | JP-A- 63079591 | 09/04/88 |
| | | US-A- 4985362 | 15/01/91 |
| | | ZA-A- 8707088 | 29/03/88 |